



Recombinant antibody constructs in cancer therapy

Peter J Hudson

Recombinant antibodies and their fragments now represent over 30% of all biological proteins undergoing clinical trials for diagnosis and therapy. The focus on antibodies as the ideal cancer-targeting reagents recently culminated in approval by the Food and Drugs Administration for the first engineered therapeutic antibodies. In the past year, important advances have been made in the design, selection and production of new types of engineered antibodies. Innovative selection methods have enabled the isolation of high-affinity cancer-targeting and antiviral antibodies, the latter capable of redirecting viruses for gene therapy applications. In other strategies for cancer diagnosis and therapy, recombinant antibody fragments have been fused to radioisotopes, drugs, toxins, enzymes and biosensor surfaces. Bispecific antibodies and related fusion proteins have been produced for cancer immunotherapy, effectively enhancing the human immune response in anticancer vaccines and T cell recruitment strategies.

Addresses

Cooperative Research Centre for Diagnostic Technologies, The Commonwealth Scientific and Industrial Research Organisation, Molecular Science Unit, 343 Royal Parade, Parkville, Victoria 3052, Australia; e-mail: peter.hudson@molsci.csiro.au

Current Opinion in Immunology 1999, 11:548-557

0952-7915/99/\$ - see front matter © 1999 Elsevier Science Ltd. All rights reserved.

Abbreviations

C	constant
CDR	complementarity-determining region
C _H	heavy-chain C-region
FACS	fluorescence-activated cell sorters
FDA	Food and Drug Administration
IFOM	imaging figure of merit
PCR	polymerase chain reaction
scFv	single-chain Fv molecule
V	variable
V _H	heavy-chain V-region
V _L	light-chain V-region

Introduction

A new wave of antibodies and antibody fragments are about to enter the clinic, for cancer diagnosis and for therapy, and the term 'engineered antibodies' will be embraced by the general public for the first time [1*,2*]. Since it takes a decade to develop a new therapeutic reagent into a viable commercial product, many of these new antibodies are considered primitive designs that were conceived approximately 10 years ago. Some are based on fusion of murine Fv fragments (these consist of complexes of variable [V]-regions from the immunoglobulin light-chain [V_L] and heavy-chain [V_H]) onto human frameworks [2*,3,4*]; others are cleverly designed resurfacing of murine antibodies, for example by grafting murine CDR (complementarity-determining region) loops onto a human antibody framework [3,5]. The most spectacular examples

of effective cancer treatment using recombinant antibodies that have recently been approved by the Food and Drugs Administration (FDA) include 'Rituxan' (IDEC Pharmaceuticals Inc., San Diego, CA) and 'Bexxar' (Beckman Coulter Inc., Fullerton, CA), both targeting CD20 for therapy of non-Hodgkin's lymphoma [4*], and 'Herceptin' (anti-Her2; Genentech, San Francisco, CA) for breast cancer [5]. The sales of these antibodies for cancer therapy are increasing rapidly, from virtually nil in 1996 to potentially over 1 billion \$US in 2001 and have revitalised the interest of the biotechnology industry.

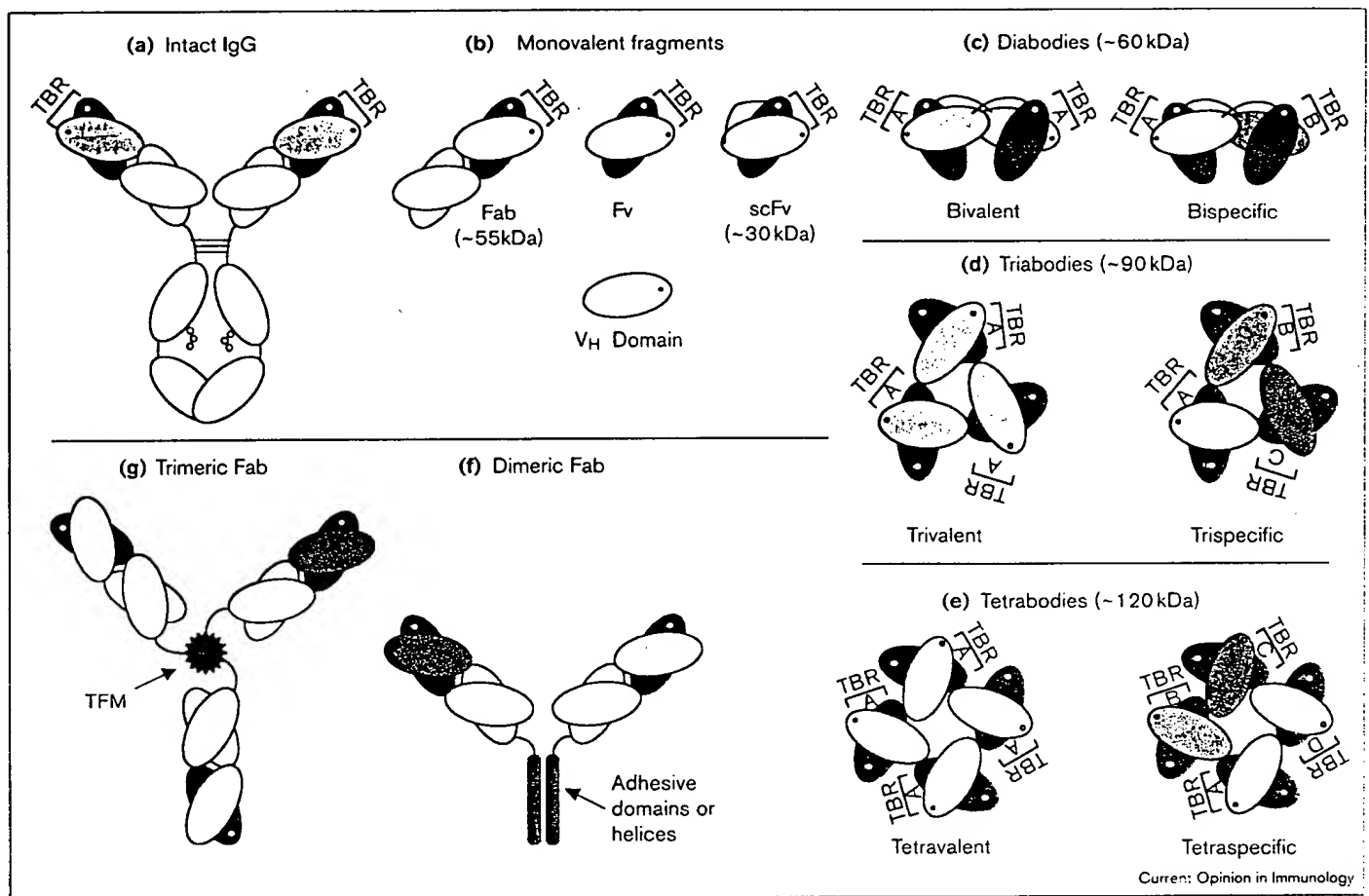
Technology for antibody design has taken enormous strides forward and new library-display and -selection procedures have made classic hybridoma technology obsolete [6**,7]. It is now possible to select high-affinity antibody fragments directly from a viral culture rather than from a live mouse. One significant advantage of this new technology is the isolation of antibodies with new binding specificities against hitherto refractory antigens, thereby avoiding the limitations inherent in the mammalian immune response [8**,9]. Antibodies are still the paradigm for the design of high-affinity, protein-based binding reagents [2*,3]. In the past year recombinant antibodies have been reduced in size, dissected into minimal binding fragments and rebuilt into multivalent, high-avidity reagents [2*,3]. Antibody fragments have also been fused with a range of molecules limited only by the imagination — including enzymes for prodrug therapy, toxins for cancer targeting, viruses for gene therapy, cationic tails for DNA delivery, liposomes for improved drug therapy and biosensors for real-time detection of target molecules. Clinical diagnostic applications of antibody fragments include the full range of *in vitro* immunoassays (30% of the diagnostic industry world-wide, worth 10 billion \$US per annum) through to *in vivo* tumour- and clot-imaging reagents.

This review describes many of the publications in the past 12 months and highlights developments in the design, production and clinical applications of recombinant antibody fragments, primarily for cancer diagnosis and therapy.

Recombinant antibodies designed for multiple valency (high avidity)

Intact antibodies are polyvalent molecules; this provides a significant increase in functional affinity (avidity) due to the simultaneous binding to two or more target antigens. Multiple binding sites effectively reduce the off-rate, thereby increasing the retention time of the antibody bound to the target antigen. IgG molecules are bivalent — with two antigen-binding Fab arms (Figure 1a) — whereas IgM molecules are pentameric (i.e. they have 10 Fab arms) and have a further increase in avidity compared with IgG.

Figure 1



Current: Opinion in Immunology

Schematic representation of recombinant antibody constructs. (a) Intact IgG (bivalent). (b) Monovalent immunoglobulin fragments (Fab, Fv, scFv and a V-domain). Also shown are scFv multimers: (c) diabodies; (d) triabodies; (e) tetrabodies. V-domains and C-domains are represented by ovals (V_H-domains are shaded grey with amino-terminal dots, V_L-domains are black and C-domains are white) and linkers are represented as black lines. Fv modules are shown with

the different target (antigen)-binding regions (TBRs)-A, -B, -C or -D and with different shading within the V_H-domains representing different target specificity. For size comparison, also shown are (f) a domain-conjugated dimeric Fab and (g) a chemically conjugated Fab trimer (using the maleimide cross-linking reagent, TFM). Only one V-domain arrangement is shown for each structure; there are obviously alternative orientations of V-domains and linker polypeptides.

The Fab and Fv modules are monovalent and represent a single antigen-binding arm of the parent immunoglobulin. The Fv module comprises aligned V_H and V_L domains and is the smallest fragment that retains the full monovalent binding affinity of the intact parent antibody (Figure 1b). Ideally, the more antigen-binding arms (higher valency) in an antibody, the higher avidity it will have to the target antigen [10]. Flexibility between antigen-binding sites is important; in intact immunoglobulins the 'elbow' angle within Fab arms and 'hinge' angles between arms allow significant flexibility and numerous cross-linking geometries [11]. Despite this flexibility, intact immunoglobulins are often not able to cross-link adjacent receptors on the same viral or cell surface [1*,12]. Recently, cross-linked homodimers of whole immunoglobulins (with four Fab arms) have been shown to cross-link adjacent receptors on the same cell surface and thereby activate intracellular signalling and apoptosis [13]. Many immunoglobulins that are specific for

cancer cells, which are currently sitting idly in laboratory freezers, are predicted to have significantly improved therapeutic value when engineered into flexible structures capable of cross-linking receptor molecules [1*,13]. However, cross-linked immunoglobulins are probably too large (e.g. 300 kDa for IgG dimers) for effective tumour penetration [14*]. Therefore, to reduce size but retain high avidity, there have been a few successful attempts to chemically cross-link Fab molecules into dimers or higher multimers (Figure 1g) to produce high-avidity reagents in the ideal 60–120 kDa size range — capable of rapid tumour penetration without fast clearance in the kidney [15,16].

Recombinant antibody constructs usually comprise single-chain Fv molecules (scFvs, ~30 kDa in size; Figure 1b), in which the V_H and V_L domains are tethered together with a polypeptide linker to improve expression and folding efficiency. As monomers, these small Fv

modules (~30 kDa) would be ideal for tumour penetration but they are monovalent (low avidity) and cannot be produced by proteolysis of intact immunoglobulins. Therefore, to increase functional affinity (avidity) and to increase the size and thereby reduce the blood clearance rates, the monomeric scFv fragments are complexed into dimers, trimers or larger aggregates using adhesive protein domains or peptide linkers. Perhaps the simplest design for a bivalent scFv dimer is a 60 kDa diabody (Figure 1c) [3,17*] in which a short five-residue linker between V_H - and V_L -domains of each scFv prevents alignment of V-domains into a single Fv module and instead results in association of two scFv molecules. Diabodies have two functional antigen-binding sites. Recently, a surprising discovery was that reduction of linkers to less than three residues was shown to prevent the formation of a diabody and instead directs three scFv molecules to associate into a trimer (90 kDa triabody; Figure 1d) with three functional antigen-binding sites [17*]. Association of four scFvs into a tetravalent tetra-body has also been reported ([18]; Figure 1e).

The gain in functional affinity through multivalent binding (avidity) makes trimeric and tetrameric scFvs particularly attractive for *in vivo* tumour imaging as an alternative reagent to diabodies and other dimeric scFv constructs [19]. Of course, multiple binding to surface-bound antigens is dependent on correct alignment and orientation in the Fv modules of diabodies and triabodies — otherwise gains in functional affinity are likely to be small. Diabodies and triabodies have been shown to be relatively flexible molecules, as judged from the orientation of antigen-binding sites revealed in single-molecule electron microscope images [20]. The effect of manipulating linker length, sequence and structure on diabody and triabody stability and flexibility can now be analysed using single-molecule imaging. The ability of multimeric (and therefore multivalent) scFvs to cross-link surface receptors is unknown and will obviously depend on flexibility between the Fv modules and the orientation of the antigen-binding sites, as well as the structure of the receptor.

Cancer targeting studies using multivalent antibody fragments

Monovalent antibody fragments (Fabs and scFvs) clear rapidly from the blood due to their small size and have relatively high off-rates due to the single binding site [14*,15,16,21]. On the other hand, whole immunoglobulins appear to be too large for effective tumour penetration and their slow clearance can result in high retention in liver and other organs [14]. The most recent studies [22**,23–25] concur that multimers in the 60–120 kDa size range, with high functional affinity (avidity), are ideal for tumour targeting. In murine xenograft models, radiolabelled diabodies (60 kDa) [22**,23,24], minibodies (here, an scFv is joined to a heavy-chain constant [C]-region 3 [C_H3]; dimers of scFv- C_H3 molecules join together via the C_H3 to form

minibodies with a size of 90 kDa) [25,26] and dimerised Fabs (120 kDa; Figure 1f) [14*,15,16] all exhibit improved tumour targeting, as measured by tumour : blood ratios, compared with monovalent scFv (30 kDa), Fab (60 kDa) or parent bivalent IgG (150 kDa). An intriguing result showed that diabodies had improved tumour targeting compared with scFvs that had been dimerised by disulphide bonds [22**]. Diabodies might therefore be more stable *in vivo* and, indeed, the polypeptide linkers in diabodies are relatively inaccessible to proteases compared with the long linkers required for monomeric scFv [17*]. The radionuclide half-life is also a significant factor in determining the ideal biodistribution ratio; thus diabodies (60 kDa) will have more rapid tumour penetration and clearance than a $(Fab)_2$ (100 kDa) and are better suited to radioimaging using radionuclides with a short half-life, like technetium (used as a γ emitter) or fluorine (used as a positron emitter). The actual benefit for tumour targeting can be estimated by an 'imaging figure of merit' (IFOM) score [24]. For short-lived radioisotopes (e.g. ^{123}I or ^{18}F), 60 kDa diabodies are ideal for clinical imaging due to the faster clearance rates and greater IFOM score compared with 90 kDa minibodies (scFv- C_H3 dimers). For longer half-life radiolabels (e.g. ^{90}Y or ^{111}In), the slower clearance of minibodies compared with diabodies generates a higher IFOM score. Following the trend, we would expect that radiolabelled triabodies (90 kDa) and tetra-bodies (120 kDa) would outperform diabodies in total tumour uptake due to higher avidity and reduced blood clearance.

Further improvements in pharmacokinetics can be achieved by increasing the surface negative charge or modifying the radionuclide chemistry, both strategies reducing the renal uptake frequently observed in small engineered antibodies [27,28]. The *in vivo* half-life of recombinant antibody constructs can also be significantly increased by conjugation to polyethylene glycol [29]. The improved functional affinity, tumour penetration and biodistribution of these novel recombinant antibody constructs are promising for the development of a new generation of reagents for tumour imaging and therapy.

Engineering antibodies with multiple specificity: the fusion of two or more antigen-binding sites

The structural requirement for multispecificity is to fuse two or more binding domains (e.g. Fv modules) together, with sufficient flexibility to allow simultaneous binding to different target epitopes. The simplest bispecific antibody is one that binds to two adjacent epitopes on a single target antigen, thereby gaining an avidity advantage to the antigen [30*,31]. However, most bispecific antibodies are designed as cross-linking reagents that bind to different target antigens [2*,3]. This has been a major focus in cancer therapy, particularly for recruitment of cytotoxic T cells [32,33], macrophages [34] or 'toxic' viruses (such as adeno-associated virus) for gene therapy [35,36]. In an

elegant and simple radiotherapy strategy, bispecific antibodies have been configured for direct recruitment of a radiolabelled chelator to the cancer site [37]. Bispecific antibodies can be produced by fusion of two hybridoma cell lines into a single 'quadroma' cell line (among the products are immunoglobulin molecules with varying mixtures of heavy and light chains from the different 'parent' cells) but this technique is complex, time consuming and often ineffective. Far simpler strategies incorporate chemical conjugation to couple two different Fab modules together (Figure 1f) [14*,15]. Recently, superb mammalian cell expression systems have been developed for the production of bispecific minibodies, using designed C_H3 interfaces that force efficient heterodimerisation of two different Fab- C_H3 molecules [38**].

Bispecific diabodies are simple recombinant constructs comprising two scFv molecules that are forced to combine together since each contains a short (five residue) linker joining V_H - and V_L -domains that prevents monomeric scFv formation [17*]. Most examples have focused on T cell recruitment; using one Fv module targeted to CD3 on T cells and with a second Fv module targeted to a cancer marker such as epithelial glycoprotein 2 (EGP-2 or Ep-CAM), carcinoembryonic antigen (CEA) or CD19 [39-41]. There are innovative alternative methods to fuse two scFvs into a bispecific conformation but the stability of these molecules *in vivo* is questionable [42,43]. Bispecific diabodies and scFv-dimers have two advantages over bispecific antibodies: the first is that, by lacking Fc domains, bispecific diabodies will only activate T cells when cross-linked to target (cancer) cells; the second advantage is that the anti-bispecific response in the host is minimised due to the small diabody size and, again, lack of Fc domains.

Other applications of bispecific diabodies include cross-linking cancer cells to serum immunoglobulin, thereby inducing the complement cascade — triggering mononuclear phagocyte respiratory burst and phagocytosis — and directing synergistic T cell cytotoxicity.

Bifunctional antibodies: fusing the target binding site to a second functional domain

The original 'magic bullet' concept is still alive; a number of cell-specific antibodies are being evaluated as delivery agents to direct a cytotoxic component to a tumour site [1*,44]. Both scFv and Fab fragments provide effective and highly specific *in vivo* targeting reagents [21,22**,23-25] but, unless conjugated to Fc domains, will not recruit the complement cascade and related cytotoxic functions. The fusion to Fc domains is a simple recombinant construct but the induction of Fc-dependent cytotoxic effector functions *in vivo* requires that the connecting hinge to Fc retains the disulphide linkage and flexibility required for effective clustering and presentation of C_H2 domains [11]. New types of immunotoxins have been produced by recombinant or chemical fusion of scFv and Fab fragments to cytotoxic drugs, radionucleotides, peptides, proteins and

liposomes [44,45,46*]. Innovative improvements to the immunotoxin concept include antibody-directed enzyme-prodrug therapy (ADEPT), a strategy of using antibody-enzyme fusion proteins to activate a prodrug into a cytotoxic agent specifically at the target cell site [47*]. Antibody-fusions have been used to target the transferrin receptor and thereby cross the blood/brain barrier, carrying epidermal growth factor (EGF)-linked radionuclides and even hammerhead ribozymes [48,49]. Recombinant antibodies have been fused to viral capsid proteins to redirect viruses as gene therapy 'delivery capsules' [50,51*] and fused to DNA-binding cationic tails to directly deliver genes [52]. ScFv-fusions displayed on T cell surfaces create tumour-specific cytotoxic 'T-bodies' [53]. ScFv-fusions to IL-2 and IL-12 have been used for cytokine stimulation and T cell proliferation at the target tumour site [54,55].

In a different therapeutic strategy, tumour antigens have been converted into vaccines by increasing their immunogenicity through targeting to antigen-presenting cells using anti-B7 antibodies [56] or by activation of T cells using anti-CD40 antibodies [57]. In a related vaccine strategy, a lymphoma-derived scFv-chemokine fusion induced a strong antitumour response [58*]. This report stressed the advantages of chemokines over cytokines in minimising the immunogenicity of the fusion component. Of course, anti-idiotypic 'mimics' are an alternative when the tumour antigen is refractory to the vaccine construction but to date this strategy has been disappointing [59].

Recombinant designs for antibody fusions are really only limited by the imagination and an expression system capable of producing correctly folded protein products. In some cases, the fusion proteins can be synthesised *in vivo* — at the site of gene (DNA) transfection [57,58*], injection or 'gene-gun' insertion of an expression plasmid into appropriate target cells.

Designing the high-affinity 'front-end' of recombinant antibodies: improvements in the 'antigen-binding surface'

The most successful strategy for affinity enhancement is to mutate Fab or scFv molecules as single monovalent domains, select for improved affinity and then conjugate the domains together into high-avidity multimers. Current dogma states that the antigen-binding surface is comprised of six CDR loops and therefore most of the designed mutations for improving affinity have been targeted to these CDR loops or the adjacent underlying residues [60,61]. Some of these mutants have spectacular affinity enhancements to cancer antigens (e.g. Her-2) [3,21]. We should note that some antibodies bind to target antigens using only four CDR loops and, if these loops can be predicted, the number of contact residues required for mutation will be significantly reduced [60]. Perhaps the only disappointment with *in vitro* affinity maturation strategies has been that designed interface

B

mutations [60,61] have been less effective than random mutation and selection using phage display [4*,21]. These strategies for phage library mutation and selection are discussed in detail below. Part of the protein-design problem is that we do not yet know how to design precisely complementary surfaces. The effect of solvating buried water molecules is still in debate and it is uncertain whether such solvation increases or decreases entropy. A rigorous analysis of antibody surfaces shows that interfaces are complementary in electrostatic potential but not, as one would expect, in charge [62].

These reports show that we still are primitive in our ability to design specific interface mutations that will result in affinity enhancement, even using relatively high-resolution structural analysis of both the free and antigen-bound antibody. The highest resolution crystal structure to date for a peptide-specific Fab, a breast-cancer-specific anti-MUC1 Fab [63], shows an unusual *cis*-Pro in its CDR3. This type of detailed structural information can lead to the design and selection (e.g. for anti-MUC1) of either high-affinity mimetics of CDR3 that target MUC1 with rapid tumour penetration or of constrained peptides that mimic the natural MUC-1 conformation as peptide vaccines.

Can V-domains be used alone as targeting reagents?

Many attempts in the 1990s to produce a single, recombinant V-domain with high affinity to a target antigen failed [2*,3]. Then, in a remarkable discovery in nature, both camelids (camels, llamas and alpacas) and sharks were found to produce antibodies that display single high-affinity V_H -domains rather than the Fv module and that therefore bind to their target antigens using just three CDR loops [64]. Perhaps the large loop size in camelid V-domains, stabilised by an intraloop disulphide bond, is a critical component in providing a sufficiently large antigen-binding surface. Recently, a human V-like domain (the extracellular domain from the T cell activation marker, CTLA4) was successfully adapted as a scaffold for display and selection using large CDR loops that can penetrate clefts in the target antigen. These humanised V-like domains may be an ideal framework for targeting clefts and canyons in receptors, enzymes and viruses [65*].

Stabilised Fv molecules and improved expression levels

The scaffold chosen for recombinant antibodies has been a simple choice between scFv or Fab molecules, with most groups favouring scFvs due to the higher expression levels compared with Fabs in bacteria although both can be expressed to over 1 gm per litre using fermenters [66]. Usually, a carboxy-terminal polypeptide tail is added for efficient affinity-purification after expression into the periplasm of *Escherichia coli*, signal peptide excision, V-domain folding and disulphide bond formation [66]. In

scFvs, the polypeptide linker must span at least 35Å (3.5 nm) between the carboxyl terminus of one V domain and the amino terminus of the other V domain without compromising the fidelity of the V_H - V_L pairing and antigen-binding site [17*]. The amino termini of both V_H and V_L are near to, but not part of, the antigen-binding interface, so there is little to choose between the two possible orientations, V_H - V_L or V_L - V_H .

For larger immunoglobulin-based designs, a number of alternative expression systems have been used — especially the yeast *Pichia pastoris* and plant expression systems [66]. However, for those scFvs and Fabs that do not express to high levels, one solution is to increase the stability of Fv modules through the addition of buffer components or the manipulation of pH and ionic strength [67]. Specific protein framework mutations to improve stability and expression levels include placing glutamine at position 6 in the heavy chain [68], design of 'knobs-into-holes' interface mutations [3,38**] or the design of interdomain disulphides [69]. Key mutations responsible for affinity enhancement are also found in the V_H/V_L interface, usually by improving folding and stability and thereby enhancing expression levels [3]. To select for optimal V-domain alignments and Fv stability, novel phage selection methods were developed — incorporating a strategy of Fv fragment stabilisation in the presence of antigen [70]. In this latter method, V_H - and V_L -domains are displayed separately followed by an antigen-selection strategy that favours high-affinity Fv molecules with strong V_H - V_L pairings.

Antibodies can be synthesised inside the cell or internalised for gene- or protein-targeted therapies. These intracellular antibodies (called 'intrabodies') have now been designed without intradomain disulphides to improve the folding efficiency in the reducing environment of the cytoplasm [71]. The application of intracellular targeting opens up a new therapeutic field for functional 'knockouts' that could rival gene silencing strategies based on antisense and ribozyme delivery.

Antibody fragment libraries: construction, display and selection

Bacteriophage libraries

Phage-display technology is currently the most popular *in vitro* method for the selection of high-affinity recombinant antibody fragments [6**,7,8**,9]. The current fd phage and phagemid vectors enable coupling of affinity selection (based on the displayed repertoires of Fab and scFv fragments) to the recovery of the packaged gene encoding that antibody. This system is highly effective and has been used extensively to isolate human Fab and scFv fragments against a wide range of cancer cell surface markers [6**,7,8**,9] and related proteins that can be targeted for cancer therapy [72]. Human antibody genes can be extracted from preimmunised donors and have the advantage of already being somatically matured to high

binding affinity (dissociation constant $[K_d] < 10^{-7}$ M) [73]. Alternatively, antibody fragments can be selected from very large 'naïve' repertoires that have been either constructed from germline V-domains or synthesised with large numbers of mutations, typically in CDR loops [8**,9]. These antibodies can also be affinity matured, in some cases to exceptional affinity ($K_d < 10^{-11}$ M) [21]. Despite these successes, fd vectors impose a series of major limitations — including gene deletion, plasmid instability, gross phage cross-contaminations and limited library size.

Polysome libraries

Polysomes are stable protein-ribosome-mRNA complexes, which can be used to replace live bacteriophages as the display vehicle for recombinant antibody fragments [74*,75]. The polysomes are formed by preventing release of newly synthesised and correctly folded protein from the ribosome. As is the case for phage display, the selection is based on protein affinity with concomitant recovery of the gene (initially as mRNA) that encodes the protein. Repertoire diversity is directed by the mRNA population. Polysome display has many advantages over phage display, including easy synthesis of very large libraries by avoiding the need for cell transfections. The main limitation is that selection requires a ribonuclease-free environment.

Library selection

Antibody fragments displayed on either phages or polysomes can be selected by binding to covalently immobilised antigen. The choice of affinity matrix can often determine the kinetics of the selected antibody gene fragment and competitive selection procedures can discriminate between mutants with as little as a two-fold affinity difference [6**]. Phage libraries can be selected against crude target mixtures, including live cells [76], although selection on tissue sections or in whole animals is very inefficient [6**]. Cell surface selection against receptor targets can be enhanced by a novel coselection method incorporating cross-linking with the receptor-specific ligand [77].

Cell surface libraries

Antibodies had been displayed on cell surfaces before the advent of viral display systems. However the selection of live cells, usually by replica plating, limited library screens of bacterial colonies spread over each agar plate to 10^7 . With the development of high-speed FACS (fluorescence-activated cell sorters) capable of sorting over 10^8 cells per hour, cell surface libraries have re-emerged as a viable alternative to phages. Both bacteria and yeast [78**] have been successfully used to display antibodies and other immunoglobulin molecules, in both cases using mutator cells to further enhance the library diversity. The selection is best achieved using a fluorescently tagged target antigen and high-speed FACS (over 10^8 cells/hour).

Affinity enhancement of antibody fragments using libraries

Current strategies all emulate the natural somatic hypermutation and selection process that occurs *in vivo* and are based on the principal of introducing genetic mutations followed by selection of the encoded antibodies that exhibit enhanced affinity [6**]. One of the most effective random mutagenesis methods incorporates replication *in vivo* using *E. coli* mutator cells to mutate phage or cell surface libraries [78**]. Mutations can be targeted to the V-domain genes by homologous gene reassortments or error-prone PCR. Mutations can be further limited to just short regions of polypeptide sequence (e.g. 5–10 residues) by specific-window mutagenesis. Usually the mutations have been directed to the CDR loops [21] but mutations in the underlying framework regions provide an alternative target for mutagenesis since they often exhibit pronounced effects on affinity, stability and expression levels [68,71]. The two-domain structure of the Fv module enables chain shuffling to be used both for sequential improvements in affinity and for humanisation [6**]. In chain shuffling, just one V-domain is altered at a time, relying on promiscuous V-domain pairing to create new functional Fv modules. For example, starting with an antibody of known target affinity, only one V-domain is displayed as a library whilst the other V-domain is held constant to provide a defined target affinity. Repeated cycles of chain shuffling and affinity selection will produce high-affinity antibodies. Using this 'guided selection' or 'epitope imprinting' strategy, it has been possible to sequentially replace the V-domains of a parent mouse antibody to form a fully human Fv with high affinity for the same epitope [6**].

New affinity maturation technologies

The fundamental problem in library mutagenesis strategies is the library size required to search the complete set of residue mutations (for example, a complete mutagenesis of just six residues requires $20^6 = 6.4 \times 10^7$ molecules). A further limitation is that the target antigen is not itself part of the maturation process but is added as a static molecule in soluble or immobilised form. Recently, an improved strategy has been developed, termed SIP (selectively infective phage) [79]. This second-generation format uncouples the display system (currently as scFv/Fab on phage heads) so that antigen/antibody recognition is an integral component of the viral transfection process [79]. In this way, continuous evolution (affinity maturation) of the antigen-antibody interaction can be theoretically achieved since affinity is now coupled to the efficiency of viral transfection. The antigen is presented as a soluble fusion molecule with the amino-terminal domain of fd-gpIII and the antibody fragment repertoires are displayed on the phage as fusions to the carboxy-terminal fd-gpIII domain. Similar to phage selection, the use of competing concentrations of antigen allows selection of the higher affinity scFvs/Fabs. The vectors and host cells are still under development and it would be unwise to suggest that SIP

B

methods will replace the established fd-phage affinity maturation procedures in the short term. A major hurdle is the presentation of antigen as a soluble, correctly folded fusion molecule [79]. If this can be solved, phage propagation *in vivo* during continuous cell culture could provide a simple selection process for a high affinity ligand-receptor interaction.

Transgenic (humanised) mice

Injections into live animals are still the simplest way to produce antibody fragments despite the facts that phage-display libraries can supersede natural immune repertoires in size and that affinity maturation strategies can supersede somatic mutation in the speed of selecting high affinity mutants. Transgenic mice (xenomice) have been produced that lack the native murine immune repertoire and instead harbour most of the human immune system V-genes in the germline [80]. Injection of these 'humanised' animals with a foreign antigen or hapten effectively evokes an immune response and a human-like antibody is produced in B cells [80]. The antibody genes can be recovered from B cells either by PCR and library selection or by fusion into a monoclonal cell line by classic hybridoma technology. It is uncertain whether humanised mice will produce only the conventional range of antibody affinities and, in that case, may require further affinity maturation using phage or polysome display. Also, and despite the commercial hype pushing the wide-ranging use of these xenomice, there are differences between murine and human glycosylation patterns (especially in Gal α 1-3) that have yet to be solved before application as *in vivo* therapeutics [81].

Conclusions

During the past 12 months there has been a rapid increase in both scientific and commercial interest in recombinant antibodies, leading to the large number of publications covered in this review. Clinical interest is due to encouraging results from Phase III trials, which have led to several recent approvals by the FDA of therapeutic antibodies for cancer. Scientific interest has stemmed from the elucidation of the key elements required for antibody-fragment design and efficient expression. Many of the antibody fragments and fusion proteins discussed in this review are now undergoing scale-up production, some in transgenic animal or plant expression systems, and we eagerly await the results of clinical trials. In the next 12 months, it is likely that we will have generic solutions for improved antibody stability — leading to improved expression yields — and generic strategies for the design of multimers, immunotoxins and other innovative fusion proteins. Library display and selection strategies have been significantly improved to the point that these *in vitro* systems are now more effective than conventional hybridoma technology. There are a number of useful Internet sites with information about recombinant antibodies and fragments [82–87]. By providing a highly stable and

protease-resistant scaffold, recombinant antibody fragments will continue to be the paradigm for selection of high-affinity cancer-targeting reagents.

Acknowledgements

I thank John Atwell, Bob Irving, Greg Coia and Alex Kortt for their comments on this review and particularly John Atwell for Figure 1.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Farah RA, Clinchy B, Herrera L, Vitetta ES: The development of monoclonal antibodies for the therapy of cancer. *Crit Rev Eukaryot Gene Expr* 1998, 8:321-345.
This review is an excellent summary of the clinical applications using monoclonal antibodies and recombinant derivatives up to 1998.
2. Hudson PJ: Recombinant antibody fragments. *Curr Opin Biotechnol* 1998, 9:395-402.
This review summarises the highlights in recombinant antibody designs in 1997 and early 1998. A comparison with my current review here demonstrates the very rapid progress that has been made in the design and engineering of recombinant antibody fragments throughout 1998 and 1999.
3. Dall'Acqua W, Carter P: Antibody engineering. *Curr Opin Struct Biol* 1998, 8:443-450.
4. Leget GA, Czuczman MS: Use of rituximab, the new FDA-approved antibody. *Curr Opin Oncol* 1998, 10:548-551.
This publication reviews the pharmacokinetics and Phase I and II trials for the first FDA-approved recombinant cancer therapeutic antibody. The antibody was designed as a simple chimera of mouse V-domains fused to human C-domains. In the pivotal study of Rituximab, an overall response rate of 50% was achieved with median time to progression in responders of 13.2 months. It is a useful reference for initial clinical data using recombinant antibodies and will be followed by many other publications in 1999 describing more detailed clinical trials with 'Rituxan' and other therapeutic recombinant antibodies.
5. Goldenberg MM: Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. *Clin Ther* 1999, 21:309-318.
6. Hoogenboom HR, De Bruine AP, Hufton SE, Hoet RM, Arends J-W, Roovers RC: Antibody phage display technology and its applications. *Immunotechnology* 1998, 4:1-20.
This review provides the most recent detailed description about the strategies used for phage display and selection and also provides details about the current in-vogue methods and troubleshooting. The construction of the authors' own phage library is described in [8**] and their successful screening methods against cell surface antigens are described in [76].
7. Bradbury A: Display technologies expand their horizons. *Trends Biotechnol* 1999, 17:137-138.
8. de Haard HJ, van Neer N, Reurs A, Hufton SE, Roovers RC, Henderix P, de Bruine AP, Arends JW, Hoogenboom HR: A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J Biol Chem* 1999, 274:18218-18230.
This is probably the largest naïve Fab library constructed to date. Refer to World Wide Web sites listed in [82–87] for descriptions of other antibody libraries that are commercially available.
9. Ridgway JB, Ng E, Kern JA, Lee J, Brush J, Goddard A, Carter P: Identification of a human anti-CD55 single-chain Fv by subtractive panning of a phage library using tumor and nontumor cell lines. *Cancer Res* 1999, 59:2718-2723.
10. Muller KM, Amdt KM, Pluckthun A: Model and simulation of multivalent binding to fixed ligands. *Anal Biochem* 1998, 261:149-157.
11. Roux KH, Strelets L, Brekke OH, Sandlie I, Michaelsen TE: Comparisons of the ability of human IgG3 hinge mutants, IgM, IgE, and IgA2, to form small immune complexes: a role for protein flexibility and geometry. *J Immunol* 1998, 161:4083-4090.
12. Hewat EA, Marlovits JC, Blaas D: Structure of a neutralizing antibody bound monovalently to human rhinovirus 2. *J Virol* 1998, 72:4396-4402.

13. Ghetie MA, Podar EM, Ilgen A, Gordon BE, Uhr JW, Vitetta ES: Homodimerization of tumor-reactive monoclonal antibodies markedly increases their ability to induce growth arrest or apoptosis of tumor cells. *Proc Natl Acad Sci USA* 1997, 94:7509-7514.
14. Colcher D, Pavlinkova G, Beresford G, Booth BJ, Choudhury A, • Batra SK: Pharmacokinetics and biodistribution of genetically-engineered antibodies. *Q J Nucl Med* 1998, 42:225-241.
An excellent review describing the practical limitations of large (150 kDa) IgGs resulting from slow clearance from the blood pool, poor diffusion from the vasculature and poor penetration through the tumour mass. Antibody valency and recombinant designs are also reviewed as are factors that are critical for the functional affinity of an antibody for a cell surface antigen or a polymeric antigen.
15. Casey JL, Pedley RB, King DJ, Green AJ, Yarranton GT, Begent RHJ: Dosimetric evaluation and radioimmunotherapy of anti-tumour multivalent Fab fragments. *Brit J Cancer* 1999, in press.
16. Humphreys DP, Vetterlein OM, Chapman AP, King DJ, Antoniow P, Suitters AJ, Reeks DG, Parton TA, King LM, Smith BJ *et al.*: F(ab')₂ molecules made from *Escherichia coli* produced Fab' with hinge sequences conferring increased serum survival in an animal model. *J Immunol Methods* 1998, 217:1-10.
17. Atwell J, Breheney KA, Lawrence LJ, McCoy AJ, Kortt AA, Hudson PJ: • ScFv multimers: length of the linker between VH and VL domains dictates precisely the transition between diabodies and triabodies. *Protein Eng* 1999, 12:597-604.
This publication provides the first detailed description of the diabody → triabody transition and explains why scFv trimers are the preferred conformation when very short polypeptide linkers between V-domains prevent diabody formation.
18. LeGall F, Kipriyanov SM, Moldenhauer G, Little M: Di-, tri- and tetrameric single chain Fv antibody fragments against human CD19: effect of valency on binding. *FEBS Lett* 1999, 453:164-168.
19. Beresford GW, Pavlinkova G, Booth BJ, Batra SK, Colcher D: Binding characteristics and tumor targeting of a covalently linked divalent CC49 single-chain antibody. *Int J Cancer* 1999, 81:911-917.
20. Lawrence LJ, Kortt AA, Iliades P, Tulloch PA, Hudson PJ: Orientation of antigen binding sites in dimeric and trimeric single chain Fv antibody fragments. *FEBS Lett* 1998, 425:479-484.
21. Adams GP, Schier R, Marshall K, Wolf EJ, McCall AM, Marks JD, Weiner LM: Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. *Cancer Res* 1998, 58:485-490.
22. Adams GP, Schier R, McCall AM, Crawford RS, Wolf EJ, Weiner LM, • Marks JD: Prolonged *in vivo* tumor retention of a human diabody targeting the extracellular domain of human HER2/neu. *Brit J Cancer* 1998, 77:1405-1412.
This phage-selected and affinity-matured recombinant antibody may be the world's first phage-engineered scFv in clinical trials. There is an excellent discussion of multivalent antibody design and an explanation of the functional affinity (avidity) gains that are made via apparently reduced off-rates, which result from both multiple binding and rebinding to the target antigens.
23. Viti F, Tarli L, Giovannoni L, Zardi L, Neri D: Increased binding affinity and valence of recombinant antibody fragments lead to improved targeting of tumoral angiogenesis. *Cancer Res* 1999, 59:347-352.
24. Wu AM, Williams LE, Zieran L, Padma A, Sherman M, Bebb GG, Odom-Maryon T, Wong JYC, Shively JE, Raubitschek AA: Anti-carcinoembryonic antigen (CEA) diabody for rapid tumor targeting and imaging. *Tumour Targeting* 1999, 4:47-54.
25. Hu S, Shively L, Raubitschek A, Sherman M, Williams LE, Wong JY, Shively JE, Wu AM: Minibody: a novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res* 1996, 56:3055-3061.
26. Olafsen T, Rasmussen IB, Norderhaug L, Bruland OS, Sandlie I: IgM secretory tailpiece drives multimerisation of bivalent scFv fragments in eukaryotic cells. *Immunotechnology* 1998, 4:141-153.
27. Pavlinkova G, Beresford G, Booth BJ, Batra SK, Colcher D: Charge-modified single chain antibody constructs of monoclonal antibody CC49: generation, characterization, pharmacokinetics, and biodistribution analysis. *Nucl Med Biol* 1999, 26:27-34.
28. Arano Y, Fujioka Y, Akizawa H, Ono M, Uehara T, Wakisaka K, Nakayama M, Sakahara H, Konishi J, Saji H: Chemical design of radiolabeled antibody fragments for low renal radioactivity levels. *Cancer Res* 1999, 59:128-134.
29. Chapman AP, Antoni P, Spitali M, West S, Stephens S, King DJ: Therapeutic antibody fragments with prolonged *in vivo* half-lives. *Nat Biotechnol* 1999, 17:780-783.
30. Robert B, Dorvillius M, Buchegger F, Garambois V, Mani JC, • Pugnieres M, Mach JP, Pelegrin A: Tumor targeting with newly designed biparatopic antibodies directed against two different epitopes of the carcinoembryonic antigen (CEA). *Int J Cancer* 1999, 81:285-291.
This paper describes the design of high-avidity antibodies by targeting two adjacent epitopes on a single target antigen. These 'biparatopic' antibodies are similar to 'CRABs', the chelating recombinant antibodies described in [31]. High avidity will require that both arms bind simultaneously without a significant loss of entropy.
31. Neri D, Momo M, Prospero T, Winter G: High-affinity antigen binding by chelating recombinant antibodies (CRABs). *J Mol Biol* 1995, 246:367-373.
32. Bauer S, Renner C, Juwana JP, Held G, Ohnesorge S, Gerlach K, Pfreundschuh M: Immunotherapy of human tumors with T-cell-activating bispecific antibodies: stimulation of cytotoxic pathways *in vivo*. *Cancer Res* 1999, 59:1961-1965.
33. Manzke O, Berthold F, Huebel K, Tesch H, Diehl V, Bohlen H: CD3xCD19 bispecific antibodies and CD28 bivalent antibodies enhance T-cell reactivity against autologous leukemic cells in pediatric B-ALL bone marrow. *Int J Cancer* 1999, 80:715-722.
34. Pullarkat V, Deo Y, Link J, Spears L, Marty V, Curnow R, Groshen S, Gee C, Weber JS: A phase I study of a HER2/neu bispecific antibody with granulocyte-colony-stimulating factor in patients with metastatic breast cancer that overexpresses HER2/neu. *Cancer Immunol Immunother* 1999, 48:9-21.
35. Miller CR, Buchsbaum DJ, Reynolds PN, Douglas JT, Gillespie GY, Mayo MS, Raben D, Curiel DT: Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res* 1998, 58:5738-5748.
36. Bartlett JS, Kleinschmidt J, Boucher RC, Samuiski RJ: Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab')₂ antibody. *Nat Biotechnol* 1999, 17:181-186.
37. Kraeber-Bodere F, Faivre-Chauvet A, Sai-Maurel C, Gautherot E, Fiche M, Campion L, Le Boterff J, Barbet J, Chatai JF, Thedrez P: Bispecific antibody and bivalent hapten radioimmunotherapy in CEA-producing medullary thyroid cancer xenograft. *J Nucl Med* 1999, 40:198-204.
38. Merchant AM, Zhu Z, Yuan JQ, Goddard A, Adams CW, Presta LG, • Carter P: An efficient route to human bispecific IgG. *Nat Biotechnol* 1998, 16:677-681.
This is probably the best expression system for minibody production. Using 'knobs-into-holes' engineering, this paper describes how two different scFv-CH₃ fusion proteins are forced to conjugate exclusively into bispecific hexamers (minibodies).
39. Helfrich W, Kroesen BJ, Roovers RC, Westers L, Molema G, Hoogenboom HR, de Leij L: Construction and characterization of a bispecific antibody for retargeting T cells to human carcinomas. *Int J Cancer* 1998, 76:232-239.
40. Holltger P, Manzke O, Span M, Hawkins R, Fleischmann B, Qinghua L, Wolf J, Diehl V, Cochet O, Winter G, Bohlen H: Carcinoembryonic antigen (CEA)-specific T-cell activation in colon carcinoma induced by anti-CD3 x anti-CEA bispecific diabodies and B7 x anti-CEA bispecific fusion proteins. *Cancer Res* 1999, 59:2909-2916.
41. Kipriyanov SM, Moldenhauer G, Strauss G, Little M: Bispecific CD3 x CD19 diabody for T cell-mediated lysis of malignant human B cells. *Int J Cancer* 1998, 77:763-770.
42. Muller KM, Arndt KM, Pluckthun A: A dimeric bispecific miniantibody combines two specificities with avidity. *FEBS Lett* 1998, 432:45-49.
43. Chaudri ZN, Bartlett-Jones M, Panayotou G, Klonisch T, Roitt IM, Lund T, Delves PJ: Dual specificity antibodies using a double-stranded oligonucleotide bridge. *FEBS Lett* 1999, 450:23-26.
44. Reiter Y, Pastan I: Recombinant Fv immunotoxins and Fv fragments as novel agents for cancer therapy and diagnosis. *Trends Biotechnol* 1998, 16:513-520.

45. Tseng YL, Hong RL, Tao MH, Chang FH: Sterically stabilized anti-idiotypic immunoliposomes improve the therapeutic efficacy of doxorubicin in a murine B-cell lymphoma model. *Int J Cancer* 1999, 80:723-730.
46. Carter G, White P, Fernie M, King S, McLean G, Titball R, Carr FJ:
 - Enhanced antitumour effect of liposomal daunorubicin using antibody-phospholipase C conjugates or fusion protein. *Int J Oncol* 1998, 13:819-825.
 The authors describe a new two-step method for targeting cytotoxic drugs to tumour cells. Antibody-phospholipase-C fusion proteins first target cancer cells, then lyse drug-loaded liposomes for a cytotoxic effect localised at the tumour. This obviously has the capacity for massive drug-loading into the tumour.
47. Shabat D, Rader C, List B, Lerner RA, Barbas CF III: Multiple event
 - activation of a generic prodrug trigger by antibody catalysis. *Proc Natl Acad Sci USA* 1999, 96:6925-6930.
 This prodrug could possibly be incorporated into very useful ADEPT (antibody-directed enzyme-prodrug therapy) strategy using bispecific constructs. The antibody 'Abzyme' component catalyses cytotoxic activation across a wide range of drug-masking chemistries because it is compatible with virtually any heteroatom. The reaction cascade is not catalyzed by any known natural enzyme and can be applied to release of anticancer drugs such as doxorubicin and camptothecin.
48. Kurihara A, Deguchi Y, Pardridge WM: Epidermal growth factor radiopharmaceuticals: ¹¹¹In chelation, conjugation to a blood-brain barrier delivery vector via a biotin-polyethylene linker, pharmacokinetics, and *in vivo* imaging of experimental brain tumors. *Bioconjug Chem* 1999, 10:502-511.
49. Hudson AJ, Normand N, Ackroyd J, Akhtar S: Cellular delivery of hammerhead ribozymes conjugated to a transferrin receptor antibody. *Int J Pharm* 1999, 182:49-58.
50. Martin F, Neil S, Kupsch J, Maurice M, Cosset F, Collins M: Retrovirus targeting by tropism restriction to melanoma cells. *J Virol* 1999, 73:6923-6929.
51. Poul MA, Marks JD: Targeted gene delivery to mammalian cells by
 - filamentous bacteriophage. *J Mol Biol* 1999, 288:203-211.
 A remarkable exploitation in which scFv-targeted bacteriophages bound mammalian cells expressing the growth factor receptor ErbB2 and then amazingly underwent receptor-mediated endocytosis. The scFv-targeted phages were isolated by selection from a phage antibody library on breast tumor cells and recovery of infectious phages from within the cell.
52. Chen J, Gamou S, Takayanagi A, Ohtake Y, Ohtsubo M, Shimizu N: Receptor-mediated gene delivery using the Fab fragments of anti-epidermal growth factor receptor antibodies: improved immunogene approach. *Cancer Gene Ther* 1998, 5:357-364.
53. Mezzananza D, Canevari S, Mazzoni A, Figini M, Colnaghi MI, Waks T, Schindler DG, Eshhar Z: Transfer of chimeric receptor gene made of variable regions of tumor-specific antibody confers anticarbohydrate specificity on T cells. *Cancer Gene Ther* 1998, 5:401-407.
54. Melani C, Figini M, Nicosia D, Luisson E, Ramakrishna V, Casorati G, Parmiani G, Eshhar Z, Canevari S, Colombo MP: Targeting of interleukin 2 to human ovarian carcinoma by fusion with a single-chain Fv of antifolate receptor antibody. *Cancer Res* 1998, 58:4146-4154.
55. Peng LS, Penichet ML, Morrison SL: A single-chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-12 bioactivity and demonstrates antitumor activity. *J Immunol* 1999, 163:250-258.
56. Timmerman JM, Levy R: Dendritic cell vaccines for cancer immunotherapy. *Annu Rev Med* 1999, 50:507-529.
57. Sotomayor EM, Borrello I, Tubb E, Rattis FM, Bien H, Lu Z, Fein S, Schoenberger S, Levitsky HL: Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through *in vivo* ligation of CD40. *Nat Med* 1999, 5:780-787.
58. Biragyn A, Tani K, Grimm MC, Weeks S, Kwak LW: Genetic fusion of
 - chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. *Nat Biotechnol* 1999, 17:253-257.
 This paper is remarkable in that therapeutic delivery of the fusion protein was achieved as a DNA vaccine. This method is likely to become far more popular due to the simplicity of DNA transfection *in vivo*.
59. Tripathi PK, Qin H, Deng S, Xu C, Bhattacharya-Chatterjee M, Foon KA, Chatterjee SK: Antigen mimicry by an anti-idiotypic antibody single chain variable fragment. *Mol Immunol* 1998, 35:853-863.
60. Dougan DA, Malby RL, Gruen LC, Kort AA, Hudson PJ: Effects of substitutions in an antibody binding surface on antigen affinity. *Prot Eng* 1998, 11:65-74.
61. Braden BC, Goldman ER, Mariuzza RA, Poljak RJ: Anatomy of an antibody molecule: structure, kinetics, thermodynamics and mutational studies of the antilysozyme antibody D1.3. *Immunol Rev* 1998, 163:45-57.
62. McCoy AJ, Epa VC, Colman PM: Electrostatic complementarity at protein/protein interfaces. *J Mol Biol* 1997, 268:570-584.
63. Dokurno P, Bates PA, Band HA, Stewart LMD, Lally JM, Burchell JM, Taylor-Papadimitriou J, Snary D, Sternberg MJE, Freemont PS: Crystal structure at 1.95 Å resolution of the breast tumour-specific antibody SM3 complexed with its peptide epitope reveals novel hypervariable loop recognition. *J Mol Biol* 1998, 284:713-728.
64. Decanniere K, Desmyter A, Lauwereys M, Ghahroudi MA, Muyldermans S, Wyns L: A single-domain antibody fragment in complex with RNase A: non-canonical loop structures and nanomolar affinity using two CDR loops. *Structure* 1999, 7:361-370.
65. Nuttall SD, Rousch MJM, Irving RA, Hufton SE, Hoogenboom HR,
 - Hudson PJ: Design and expression of soluble CTLA-4 variable domain as a scaffold for the display of functional polypeptides. *Proteins* 1999, 36:217-227.
 The first reported exploitation of a soluble humanised V-like domain to target clefts and canyons in enzymes, viral capsids and receptors; in this case the target was the G-protein-coupled somatostatin receptor.
66. Verma R, Boleti E, George ATJ: Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. *J Immunol Methods* 1998, 216:165-181.
67. Worn A, Pluckthun A: Different equilibrium stability behavior of scFv fragments: identification, classification, and improvement by protein engineering. *Biochemistry* 1999, 38:8739-8750.
68. Langedijk AC, Honegger A, Maat J, Planta RJ, van Schaik RC, Pluckthun A: The nature of antibody heavy chain residue H6 strongly influences the stability of a VH domain lacking the disulfide bridge. *J Mol Biol* 1998, 283:95-110.
69. Almog O, Benhar I, Vasmatzis G, Tordova M, Lee B, Pastan I, Galliland G: Crystal structure of the disulphide-stabilised Fv fragment of anticancer antibody B1. *Proteins* 1998, 31:128-138.
70. Jager M, Pluckthun A: Folding and assembly of an antibody Fv fragment, a heterodimer stabilized by antigen. *J Mol Biol* 1999, 285:2005-2019.
71. Ramm K, Gehrig P, Pluckthun A: Removal of the conserved disulfide bridges from the scFv fragment of an antibody: effects on folding kinetics and aggregation. *J Mol Biol* 1999, 290:535-546.
72. Pini A, Viti F, Santucci A, Carnemolla B, Zardi L, Neri P, Neri D: Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J Biol Chem* 1998, 273:21769-21776.
73. Mao S, Gao C, Lo CH, Wirsching P, Wong CH, Janda KD: Phage-display library selection of high-affinity human single-chain antibodies to tumor-associated carbohydrate antigens sialyl Lewis^x and Lewis^y. *Proc Natl Acad Sci USA* 1999, 96:6953-6958.
74. Hanes J, Jermutus L, Schaffitzel C, Pluckthun A: Comparison of
 - Escherichia coli and rabbit reticulocyte ribosome display systems. *FEBS Lett* 1999, 450:105-110.
 The first detailed comparison on the use of prokaryotic versus eukaryotic ribosomes for the display of antibody-fragment repertoires. Both methods are efficient, with the authors preferring their own in-house prokaryotic system. Other groups have exploited the alternative eukaryotic ribosome methods (see [75]).
75. He M, Taussig MJ: Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for *in vitro* display and evolution of antibody combining sites. *Nucleic Acids Res* 1997, 25:5132-5134.
76. Hoogenboom HR, Lutgerink JT, Pelsers M, Rousch M, Coote J, van Neer N, de Bruine A, van Nieuwenhoven FA, Glatz JFC, Arends J-W: Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. *Eur J Biochem* 1999, 260:774-784.
77. Osbourn JK, Eamshaw JC, Johnson KS, Parmentier M, Timmermans V, McCafferty J: Directed selection of MIP-1 alpha neutralizing CCR5

antibodies from a phage display human antibody library. *Nat Biotechnol* 1998, 16:778-781.

78. Daugherty PS, Chen G, Olsen MJ, Iverson BL, Georgiou G: Antibody affinity maturation using bacterial surface display. *Prot Eng* 1998, 11:825-829.

This method has the potential to replace phage display. The library selection systems are easy, using FACS, and the method can be adapted to mutator cells for rapid affinity maturation.

79. Spada S, Honegger A, Pluckthun A: Reproducing the natural evolution of protein structural features with the selectively infective phage (SIP) technology. The kink in the first strand of antibody kappa domains. *J Mol Biol* 1998, 283:395-407.
80. Yang XD, Jia XC, Corvalan JR, Wang P, Davis CG, Jakobovits A: Eradication of established tumors by a fully human monoclonal antibody to the epidermal growth factor receptor without concomitant chemotherapy. *Cancer Res* 1999, 59:1236-1243.

81. Borrebaeck CAK: Human monoclonal antibodies: the emperor's new clothes? *Nat Biotechnol* 1999, 17:621.
82. The Recombinant Antibody Page on World Wide Web URL: <http://www.mgen.uni-heidelberg.de/SD/SdscFvSite.html>
83. The V base database of human antibody genes on World Wide Web URL: <http://www.mrc-cpe.cam.ac.uk/imt-doc>
84. The antibody Resource Page on World Wide Web URL: <http://www.antibodyresource.com>
85. Index of articles by Pharma Biotech on World Wide Web URL: <http://www.medicine-news.com>
86. Morphosys AG Combinatorial Biology in Drug Discovery on World Wide Web URL: <http://www.morphosys.com/>
87. Cambridge Antibody Technology Group Therapeutic Products on World Wide Web URL: <http://www.catplc.co.uk/>

B